Altered gene expression profiles in nasal respiratory epithelium reflect stable versus acute childhood asthma

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Background: Asthma is the most common chronic disease of childhood and has a strong genetic component.
Objective: To identify gene expression signatures that reflect asthma-related processes and to determine whether these genes were similar or distinct between stable asthma and acute exacerbations in childhood, we profiled gene expression patterns in nasal respiratory epithelial cells.
Methods: Children who had stable asthma (asthma-S; n = 10) and children experiencing an asthma exacerbation (asthma-E; n = 10) were recruited along with nonatopic children without asthma (n = 10). RNA was prepared from nasal respiratory epithelial cells isolated from each child, initially analyzed as pooled samples from the 3 groups, and further validated by using microarrays and RT-PCR with individual patient samples.
Results: Distinct gene clusters were identifiable in individual and pooled asthma-S and asthma-E samples. Asthma-E samples demonstrated the strongest and most reproducible signatures, with 314 genes of 34,886 measured as present on the chip demonstrating induction or repression of greater than 2-fold with $P < .05$ in each of 4 individual samples. Asthma-S–regulated genes encompassed genes that overlapped with those of asthma-E but were fewer (166) and less consistent with respect to their behavior across the asthma-E patient samples.
Conclusion: Exacerbated asthma status is readily distinguished based on the occurrence of strong gene expression signatures in nasal epithelial samples. Stable asthma status also exhibits differential signatures. The results suggest that there are independent gene expression signatures reflective of cells and genes poised or committed to activation by an asthma attack.
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Key words: *Asthma, childhood, microarray, genetics*

Considerable effort is taking place to identify the genes important in the development of asthma. Microarray technology can be used to identify gene profiles associated with disease states as well as novel genes for investigation or to confirm the importance of genes identified by association or linkage studies. It has been used successfully in mouse models[1,2] and other animal models[3] of asthma; however, it is more challenging to apply these techniques to human studies for several reasons. It is difficult to obtain relevant tissue and sufficient quantities of tissue for the analysis, especially during the time that the asthma is active. These challenges are magnified in children, in whom the studies are most relevant, because asthma often has its roots in childhood. Previous studies using microarray analysis have been performed by using RNA isolated from PBMCs from adults with asthma.[4] These studies are useful but do not identify relevant genes that are activated in the lungs of a patient with asthma. Another approach that has been used is to study human airway cells treated with IL-4 and/or IL-13 and to use chip array technology to determine epithelial genes that are regulated by these cytokines, which are known to be critical for the pathogenesis of atopic disorders.[5,6]

In this study, we used microarray technology in a group-averaged approach and confirmatory RT-PCR at an individual patient level to gain insight into global gene expression profiles in respiratory epithelial cells derived from the nasal mucosa of normal children and children with asthma. Children with stable asthma (asthma-S) were contrasted with those experiencing an asthma exacerbation (asthma-E) by using control samples from children without atopy or asthma. These data suggest that both stabilized and acutely affected children with asthma exhibit characteristic expression profiles that will make it possible to understand disease status and treatment response better and to suggest new therapies.

Abbreviations used
Asthma-S: Group with stable asthma
Asthma-E: Group experiencing an asthma exacerbation

METHODS

Subjects

After institutional review board approval was obtained, healthy children and children with asthma (stable or wheezing) and their

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parents attending the hospital clinics and emergency department in Cincinnati Children's Hospital Medical Center were invited to participate in the study. Asthma was diagnosed in accordance with American Thoracic Society criteria.[7,8] Participants were included in 1 of 3 groups. Stable allergic asthma (asthma-S group; N = 10) inclusion criteria were (1) age younger than 18 years, (2) physician-diagnosed asthma currently stable (not wheezing), and (3) positive skin prick test result to any of the allergens from an environmental panel that included dust mite, molds, cat, dog, feathers, weeds and ragweed, tree pollens, and grass allergen extracts (Hollister-Stier Laboratories, Spokane, Wash). Exacerbation of asthma (asthma-E group; N = 10) included children acutely wheezing with inclusion criteria similar to those of the asthma-S group with the exception of skin test positivity (skin testing was not performed in this group because it could further deteriorate the acute status of asthma). Healthy children (control group; N = 10) inclusion criteria were (1) age younger than 18 years, (2) healthy with no acute infections or major chronic illnesses, and (3) negative results to the environmental skin test prick panel. Exclusion criteria to participate in the study included (1) age 18 years or older, (2) use of nasal or systemic steroids within the last 30 days, (3) nasal malformations/tumors, and (4) acute infectious disease present in the asthma-S or control groups. Notably, many children had concurrent diagnoses of allergic rhinitis, but they were excluded if they had used nasal steroids within 30 days of the study. The use of inhaled steroids was not interrupted for this study. Skin prick testing was performed in the asthma-S and control groups by using DermaPiks (Greer Laboratories, Lenoir, NC). Histamine (1 mg/mL) and normal saline (0.9% NaCl) were used as positive and negative controls. Reactions were considered positive if there was an erythematous base with a wheal $\geq$3 mm in diameter.

Nasal respiratory cell sample collection and processing

Nasal mucosa sampling was performed by using a CytoSoft Brush (Medical Packaging Corp, Camarillo, Calif), and the sample was immediately taken to the laboratory for processing. Samples from children in the asthma-E group were taken within 1 hour of arrival to the emergency department and before any steroids were given. The cells were suspended in PBS, and an aliquot was stained with Diff-Quick (Dade Behring, Newark, Del). Cell counting was performed in 5 high-power fields, and the relative percentages of cell types were calculated. In addition, a total eosinophil cell count was performed.

RNA extraction

RNA was isolated from the nasal mucosa sample by using TRIZOL according to the manufacturer instructions (TRIZOL Reagent, Invitrogen Corp, Carlsbad, Calif). Average RNA yield was 42.6 μg. Two micrograms of RNA from each subject were pooled to form a group sample containing 20 μg. These were then submitted to the Affymetrix GeneChip Core Facility at Cincinnati Children's Hospital Medical Center for microarray hybridization using the HG-U133A GeneChip (Affymetrix, Santa Clara, Calif) according to Affymetrix guidelines (https://www.affymetrix.com/support/technical). The HG-U133A chip microarray has a total of 22,215 probe sets (excluding controls) that identifies 14,285 genes, of which 12,735 are known.[9] In addition, 4 individual samples from each group (control, stable asthma, and acute asthma) were submitted to the Affymetrix GeneChip Core Facility for microarray hybridization using the HG-U133_plus 2 GeneChip (Affymetrix) according to Affymetrix guidelines (https://www.affymetrix.com/support/technical). The HG-U133_plus 2 chip microarray has a total of 54,675 probe sets.

Microarray data analysis

Scanned output files were analyzed by using Microarray Suite 5.0 software (Affymetrix). From cell image data files, gene transcript levels were estimated as the signal strength by using the MAS5.0 (Affymetrix). Global scaling was performed to compare genes from chip to chip. Arrays were scaled to the same target intensity value (1500) per gene and analyzed independently.

Second-stage data analyses were performed by using GeneSpring software (Silicon Genetics, Redwood City, Calif). Initially, data from the pooled asthma-S and asthma-E were analyzed by looking for genes that were most different in expression relative to the control group sample. A total of 299 cDNAs corresponding to genes that were 3-fold upregulated or downregulated in the asthma-S or asthma-E group when compared the control group were identified. These 299 highly expressed cDNAs were subjected to hierarchical clustering as implemented in the GeneSpring Program[10-12] to group genes according to their relative behavior in Asthma-E and Asthma-S samples. We performed clustering of the data set by using several different normalization methods. The use of raw ratios of hybridization versus reference or the log2 of these ratios provides an assessment of genes based on their levels of expression. These 299 highly expressed cDNAs were clustered according to their relative expression profiles by using normalization of raw ratios or the log2 of these ratios to the expression of the control group. The number of immune-related genes was obtained for each cluster.

GeneChip data from the individual RNA samples were examined by a statistical approach that was designed to test the hypothesis that genes whose expression was altered in the individual samples would parallel that observed in the pooled samples. Next-generation human HG-U133_plus2 GeneChips (human HG-U133_plus2) were analyzed using MicroArraySuite 5 (Affymetrix, Santa Clara, Calif), and the resulting GeneChip intensities were exported to GeneSpring 7.0 and normalized to the median expression level among the 4 control samples. Genes whose expression varied according to diagnostic group were obtained by the following strategy. First, we selected genes that the Affymetrix algorithm reported to be present in at least 2 gene chips. This returned 32,435 genes from the 54,675 on the chip. Next, we generated an approximately normal distribution of gene expression values by representing each gene's expression as the log of its expression signal as measured by Microarray Suite. Second, ANOVA was applied to the conditions with a probability of less than .01 (acute vs stable vs control) to obtain genes differentially expressed between conditions in at least 3 out of 5 chips. Each of these lists was further filtered for median expression being at least 2-fold different between the 2 conditions. Thus, a stepwise filtering method was used to derive a total of 1378 genes whose expression was significantly different between the groups (ANOVA; $P < .01$) and was of a sufficient magnitude to increase or decrease by at least 2-fold in 3 of the 4 individual samples. The resulting gene list of 1378 genes was then subjected to cluster analysis by using standard correlation as implemented in GeneSpring.

RT-PCR analyses

Gene-specific primers (designed by using Beacon Designer software [Applied Dynamics International, Ann Arbor, Mich]) were chosen to span at least 1 intron in the genomic sequence to enable the mRNA-derived product to be distinguished from any possible contaminating genomic product. The sequences of primers for the target genes were as follows: lymphotactin (NM_003175) AATCAAGACCTACACCATCAC (sense) and TTCCTGTCCATGCTCCTG (antisense); H4 receptor (NM_021624) GGTGTGATCTCCATTCCTTTG (sense) and GCCACCATCAGAGTAACAATC (antisense); retinoic acid receptor α (hCT2294851) AGGAGACTGAGATTAGC (sense) and AAGAAGAAGGCGTAGG (antisense);

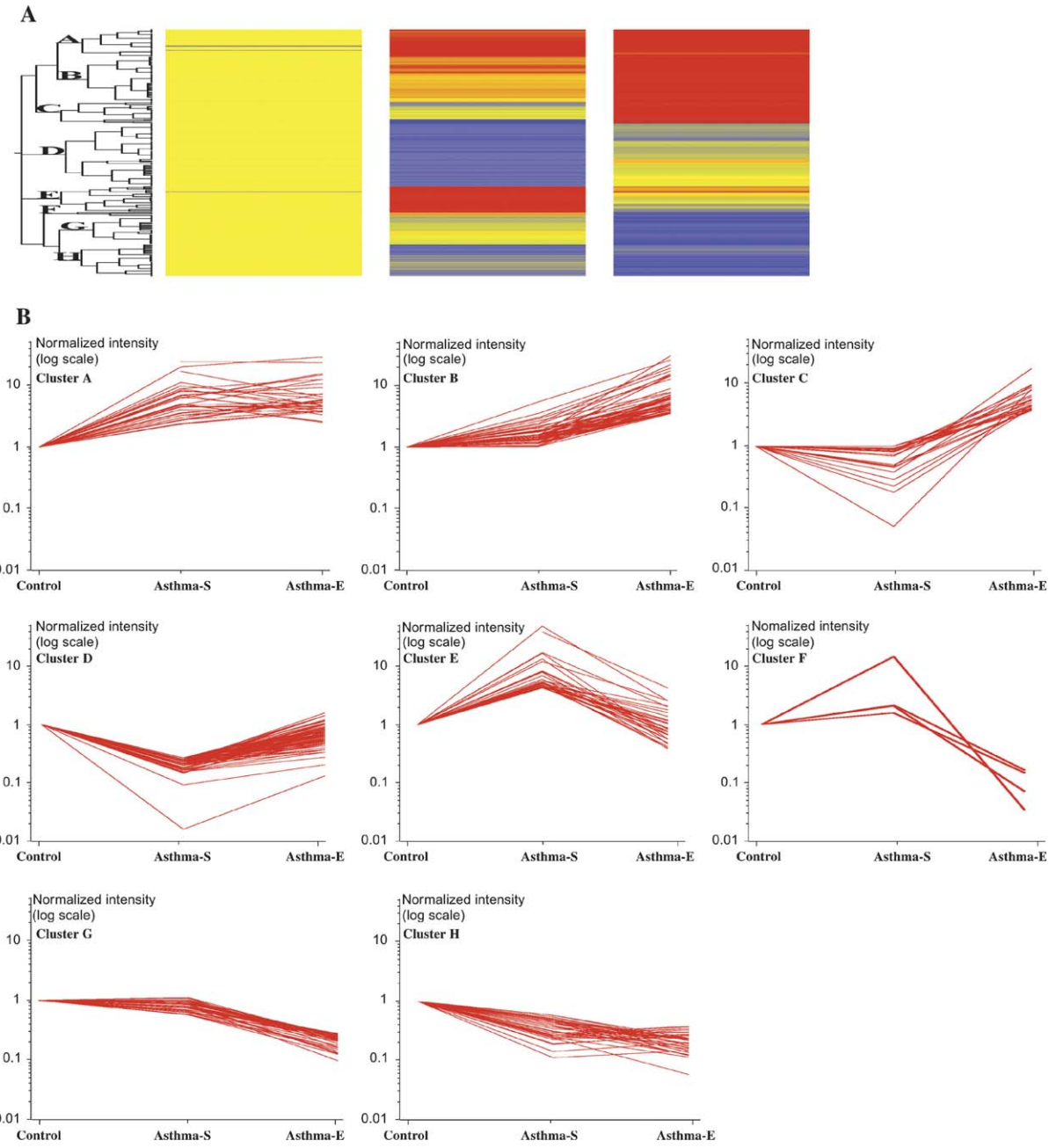

FIG 1. A, Hierarchical clustering of genes highly expressed in childhood asthma. Colors are graded to indicate increased *(red)* or decreased *(blue)* expression relative to reference. **B**, Relative expression in a given cluster. The *y-axis* represents expression normalized to control expression.

CXCL11 (NM_005409) GCTACAGTTGTTCAAGGCTTCC (sense) and TTGGGATTTAGGCATCGTTGTC (antisense); and ubiquitin C (M26880) ATTTGGGTCGCGGTTCTTG (sense) and TGCCTTGACATTCTCGATGGT (antisense). Before cDNA synthesis (using SuperScript II, Rnase H^-; Invitrogen), 1 to 2 μg of each RNA sample was pretreated with DNase I (Invitrogen). RT-PCR analysis was conducted with the iCycler (Bio-Rad, Hercules, Calif) by using the iQ SYBR Green Supermix Taq polymerase mix (Bio-Rad). The amount of double-stranded DNA product, indicated by SYBR Green fluorescence, was measured at the end of each extension cycle. The relative message levels of each target gene were normalized to the housekeeping gene, ubiquitin C, as previously described.[13,14]

Statistical analyses

Statistical differences between the relative expression levels of genes among the different groups were determined by ANOVA (1-way) of the means and SE values. This was followed by the Bonferroni procedure to allow for multiple comparisons. A P value of $<.05$ was considered significant.

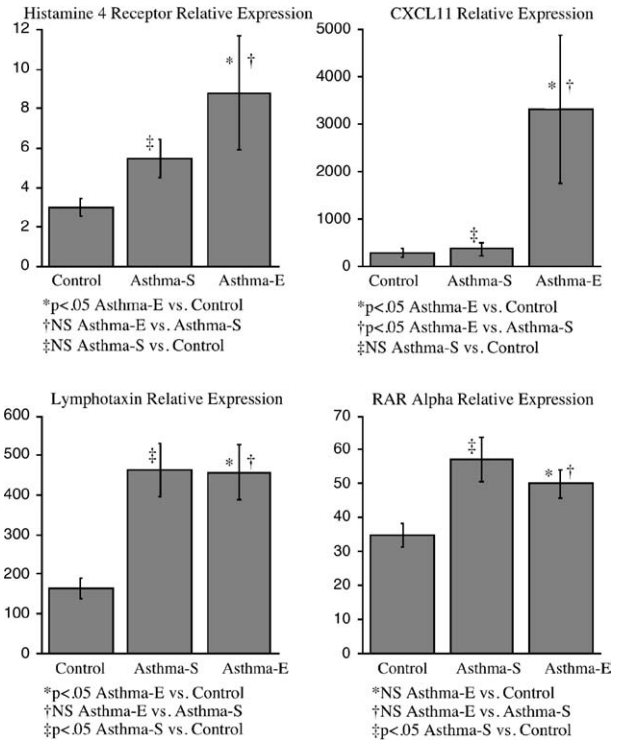

FIG 2. Quantitative RT-PCR analysis of H4R, CXCL11, lymphotactin, and retinoic acid receptor α. The *y-axis* in each graph represents relative message levels, normalized to the average of duplicate ubiquitin C message levels.

RESULTS

Subjects

The mean age in years for the control (n = 10), asthma-S (n = 10), and asthma-E (n = 10) group subjects was 11.7 (SD ± 2.3), 11.4 (SD ± 3.4), and 10.1 (SD ± 6.17), respectively. The sex (male:female) and race (African American:white) ratios were 7:3 and 8:2 for the control group, 7:3 and 5:5 for the asthma-S group, and 6:4 and 9:1 for the asthma-E group. There was no statistical difference between the groups. The children in the asthma-S and asthma-E were predominantly African American boys, which agrees with published data regarding the race and sex distributions of childhood asthma in urban environments.[15-17] The cellular composition of the nasal respiratory epithelial sample was determined for each subject. For the control group, the average number of cells per high-power field (400×) was 265 (SD ± 104), with 97.7% epithelial cells, 1.84% PMNs, 0.36% squamous cells, and 0.07% eosinophils. For the asthma-S group, the average number of cells per high-power field (400×) was 219 (SD ± 87), with 96.3% epithelial cells, 3.32% PMNs, 0.30% squamous cells, and 0.09% eosinophils. For the asthma-E group, the average number of cells per high-power field (400×) was 154 (SD ± 69), with 92.3% epithelial cells, 7.24% PMNs, 0.18% squamous cells, and 0.25% eosinophils. Epithelial cells represented greater than 92% of the total cells isolated in all groups. Not surprisingly, there were higher percentages of PMNs and eosinophils in the samples derived from children experiencing an asthma exacerbation; however, they remained minor populations (7.2% and 0.25%, respectively) compared with the respiratory cells (92.3%), and the differences were not statistically significant. A lower average of total cells was recovered from the nasal samples of children experiencing an asthma exacerbation, most likely a result of excessive mucus. RNA was isolated from each sample with an average yield of 42.6 μg per sample. Two micrograms of RNA were pooled from each subject in each group, and the 3 pools were subjected to microarray analysis (HG-U133A Affymetrix GeneChip).

Identification of 8 distinct gene clusters that are differentially regulated in stable and acute childhood asthma

In the asthma-S and asthma-E groups, the expression of only 253 (2.0%) of the known genes changed by at least 3.0-fold in either group. The mean raw expression of these genes was 2076 (SD, 4322) with a range of 17.4 to 67,068 and a median and mode of 1192 and 1903, respectively. The microarray data are summarized in Fig 1. Cluster analysis examining gene profiles revealed 8 distinct clusters of genes regulated in stable and acute childhood asthma. The genes in cluster or group A (N = 33) were similarly upregulated in both asthma-S and asthma-E. In cluster B (N = 55), genes were upregulated in asthma-S and were found to be further induced in the asthma-E group. Cluster C genes (N = 25) were unchanged in

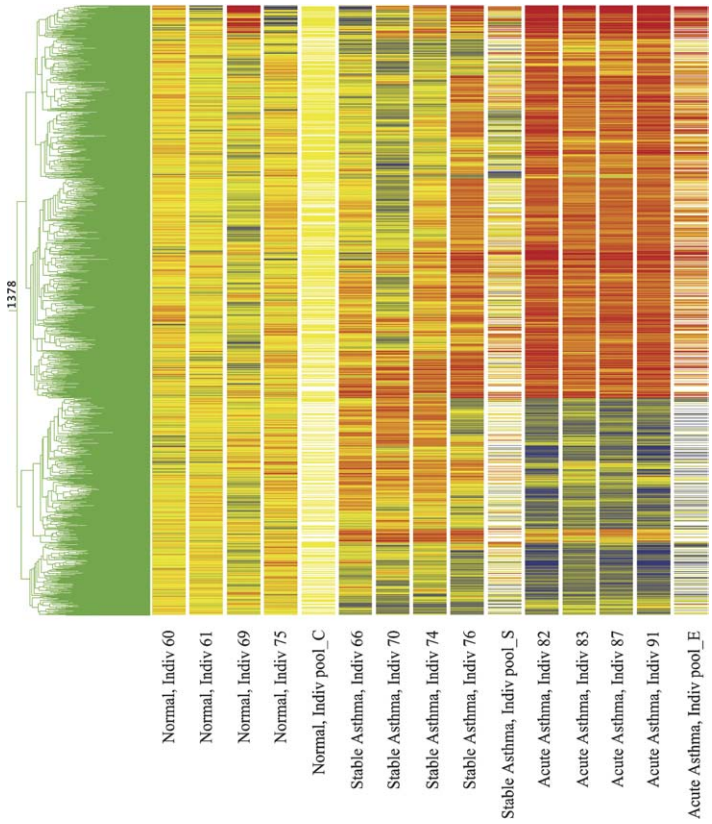

FIG 3. Hierarchical clustering of genes highly expressed in individual children with stable and acute asthma compared with controls. Colors are graded to indicate increased *(red)* or decreased *(blue)* expression relative to reference.

asthma-S but upregulated in asthma-E, cluster D genes (N = 77) were downregulated in asthma-S but unchanged in asthma-E, cluster E genes (N = 31) were upregulated in asthma-S but unchanged in asthma-E, cluster F genes (N = 4) were upregulated in asthma-S but downregulated in asthma-E, cluster G genes (N = 35) were unchanged in asthma-S but downregulated in asthma-E, and cluster H genes (N = 39) were downregulated in both asthma-S and asthma-E.

Cluster A (N = 33), representing genes similarly upregulated in both asthma-S and asthma-E, contained 32 known genes (0.25% of the total known genes). Interestingly, 27.3% of these genes were immune-related, and 21.2% were involved in signal transduction. The genes in cluster B that were upregulated during acute asthma exacerbations (asthma-E) to a higher extent than in asthma-S were 43.6% immune-related genes. Similarly, the genes in cluster C that were found to be upregulated during acute asthma exacerbations (asthma-E) but unchanged in stable asthma were 44% immune-related genes. In contrast, clusters D to H were each composed of less than 6.5% immune-related genes. Interestingly, the genes in cluster E upregulated in children with stable asthma but unchanged during acute asthma exacerbations included only 6.5% immune related genes. The genes in this profile included mainly signal transduction genes and cell function enzymes. Clusters D, F, G, and H, which contained genes that were downregulated, consisted largely of genes involved in basic cell functions and unknown genes.

RT-PCR analyses

RT-PCR analysis was used to confirm expression of genes identified by microarray. For the microarray analysis, we pooled equivalent amounts of RNA from individuals in each study group. To validate this approach, all of the individual RNA samples from the control (N = 10), asthma-S (N = 10), and asthma-E (N = 10) groups were analyzed by RT-PCR for expression of each gene. We examined 4 genes: 1 induced in the asthma-E group exclusively (cluster C), 1 induced in the asthma-S group exclusively (cluster E), and 2 simultaneously induced in both groups (cluster A). The genes examined were CXCL11, retinoic acid receptor, histamine-4 receptor, and lymphotactin (Fig 2). RT-PCR confirmed increased expression of the selected genes in asthma-S and/or asthma-E. In the case of CXCL11, expression was increased in asthma-E to a greater extent than asthma-S, as expected. In addition, the retinoic acid receptor was induced in asthma-S but not asthma-E, and lymphotactin was induced equally in asthma-S and asthma-E, as expected. Thus, the RT-PCR data validated

FIG 4. Upregulated immune-related genes **(A)** and downregulated cilia-related genes **(B)** compared with normal controls in at least 3 out of 4 samples. Colors are graded to indicate increased *(red)* or decreased *(blue)* expression relative to reference.

the genes identified by chip array and confirmed differential expression in the asthma-E and asthma-S groups.

Analyses of individual samples reveal distinct gene profiles for stable and acute asthma

To assess interindividual variation and confirm further the validity of the gene profile data obtained from the pooled samples, we performed chip array analyses on 4 individual RNA samples from each of the 3 groups: control, stable asthma, and acute asthma. We used a stepwise filtering method to derive a total of 1378 genes whose expression was significantly different between the groups (ANOVA; $P < .01$), and was of a sufficient magnitude to increase or decrease by at least 2-fold in 3 of the 4 individual samples. The gene clustering data are presented in Fig 3. The clustering results from the individual samples are presented alongside the data from the RNA samples pooled from each group (N = 10). As shown, the data from the individual samples are largely consistent both between individuals and compared with the pooled sample data. Note that between the time that the chip array analyses were performed on the pooled samples and the individual samples, the number of genes represented on the Affymetrix chip was increased. Despite the fact that more genes are assessed in the individual samples, the agreement between the individual and pooled data is striking, especially in the acute asthmatic group. In this

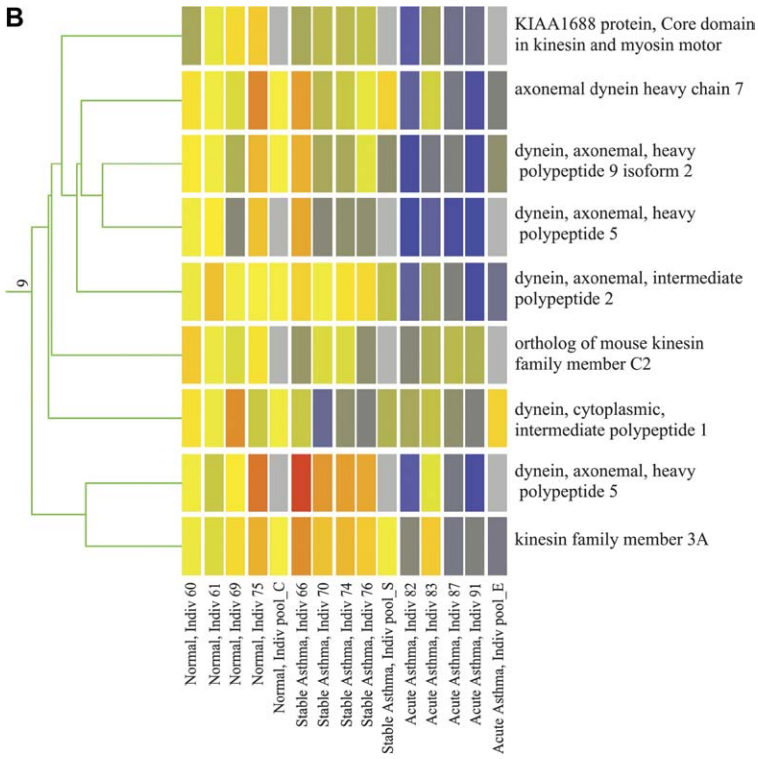

FIG 4. *Continued*

group, 887 genes were upregulated and 491 were downregulated compared with the control group. Among the 161 most upregulated and downregulated genes (at least 3-fold change; $P < .01$) that were consistent in at least 3 of the 4 samples, we searched for classes of genes to determine whether a particular class of genes was overrepresented. There were 2 classes of genes that were significant. Among the upregulated genes, we found 37 immune-related genes that were consistently upregulated at least 3-fold (Fig 4, *A*). Among the downregulated genes, we found 9 cilia-related genes that were consistently downregulated at least 3-fold (Fig 4, *B*).

DISCUSSION

Microarray technology has been used previously to identify gene profiles associated with disease states, including asthma, but previous studies have been limited to adult patients and to RNA derived from PBMCs. Because asthma most often begins in childhood, the genes identified in adults may not represent genes important for the development of asthma, but rather maintenance genes. Herein, we applied microarray technology to childhood asthma to elucidate gene profiles associated with asthma. We sought to determine (1) what gene profiles are associated with stable and acute asthma in childhood, and (2) whether the same genes that are induced during stable asthma are expressed during asthma exacerbations, or whether a distinct set of genes is activated during asthma exacerbation. Our data provide detailed profiles of nasal respiratory epithelial gene expression in pooled and individual samples from children with stable asthma and children experiencing an acute exacerbation of their asthma compared with nonatopic children without asthma. Strikingly, distinct sets of genes were activated during stable versus acute asthma. Our data establish that exacerbated asthma status can be readily distinguished based on the occurrence of strong gene expression signatures in nasal epithelial samples. Stable asthma status also exhibits differential signatures but with more variability, suggesting clinical and or mechanistic heterogeneity among the patients.

Human studies are often limited by the ability to access relevant tissue for analysis. We used cells derived from the nasal mucosa of children. The cells were largely composed ($\geq$92% in all groups) of respiratory epithelial cells. Although these cells may not fully represent the genes that are expressed in the lungs of children with asthma, they serve as an accessible alternative proxy for lower respiratory epithelium.[18-21] In addition, our approach does not detect genes that may be induced in other cells in the airways of children with asthma; only epithelial cells are being sampled, and the entire mucosa and underlying cells are likely involved *in vivo*. Nevertheless, our microarray approach and data are validated by our findings that many of the genes that we found to be induced in childhood asthma have been implicated in the pathogenesis of asthma in other studies, including arginase,[2] Suppressor of Cytokine Signaling 3,[22] complement 3a receptor,[23-25]

and lymphotactin.[26,27] For the chip array analysis, we used both pooled samples derived from equivalent amounts of RNA from each individual participant in each group, as well as individual samples. The gene expression signatures obtained from the individual samples agreed well with the pooled samples. Furthermore, RT-PCR of the individual RNA samples confirmed the chip array data.

In each cluster, we examined the percentage of immune-related genes. Interestingly, clusters that included genes either induced specifically in asthma exacerbations (cluster C) or induced at a higher level during asthma exacerbations (cluster D) contained the highest percentages (and absolute numbers) of immune-related genes. This was confirmed by further chip array analyses of the individual samples. The immune-related genes were overrepresented among the most upregulated genes. Genes that are not classified as immune genes may have direct or indirect effects on the immune system. Asthma-E samples demonstrated the strongest and most reproducible signatures, and these signatures were distinct from asthma-S. Interestingly, among the most downregulated genes, cilia-related genes were overrepresented. Because the respiratory epithelium is often damaged in asthma and there is an overproduction of mucus, one might predict that the genes important in ciliary function would be induced, so this was a surprising finding. This downregulation in cilia-related genes may contribute to asthma pathogenesis by impairing mucus clearance. Alternatively, the downregulation of cilia genes might be a response to damage and important for repair or remodeling.

Each cluster identifies novel potential target candidate genes for childhood asthma. In cluster A, the histamine 4 receptor gene was induced nearly 10-fold. This gene was recently cloned and found to be expressed in leukocytes including eosinophils, as well as the lung.[28,29] Although the biologic function of the histamine 4 receptor is not known, our data support that it is important in childhood asthma. In contrast, the histamine 1, 2, and 3 receptors were not induced in this study. Also in cluster A, SOCS-3 was induced nearly 9-fold. SOCS-3 expression was recently found to correlated strongly with the pathology of asthma and atopic dermatitis as well as serum IgE levels in allergic human patients.[22] In cluster B, the gene for the complement 3a receptor 1 was induced. In a previous study examining the role of C3a in asthma, C3a levels were increased after segmental airway challenge in sensitized adults with asthma.[23] Our data support that the receptor is also regulated during the effector phase of asthma. Several IFN-induced proteins were also induced in this cluster of genes induced in asthma-E to a greater level than asthma-S. Because asthma exacerbations in children can be associated with upper respiratory viral infections, some of these may represent an IFN-mediated antiviral response. Genes that were induced exclusively during asthma exacerbations (cluster C) included integrin α4 as well as several chemokines and chemokine receptors. Integrin α4 (CD49d), which is important for eosinophil survival and recruitment,[30,31] was induced 8.6-fold in children experiencing an asthma exacerbation, but not in children with stable asthma. In contrast, genes that were induced in asthma-S but not asthma-E (cluster E) did not include chemokine receptors or chemokines. The most strongly induced gene in this cluster was the gene encoding retinoic acid receptor α, which was induced approximately 28-fold compared with children without asthma. Retinoids exert multiple effects on lung differentiation and growth, and this receptor may contribute to lung repair or remodeling in children with ongoing stable asthma. Another interesting gene in this cluster is arginase. In a recent study, adults with stable asthma were found to have increased arginase expression in their lungs and bronchoalveolar lavage fluid compared with normal controls.[2] Our data support that arginase is also an important mediator of childhood asthma. In a previous study using microarray analysis to identify genes important in asthma, RNA isolated from peripheral blood mononuclear cells from adults with atopic asthma, allergic rhinitis (but no asthma), and healthy controls was analyzed.[4] They found decreased levels of IFN-α/β receptor (ratio 0.42), similar to our results (cluster H).

In summary, we identified distinct gene expression profiles in nasal respiratory epithelial cells of children with stable asthma (asthma-S) and children experiencing an asthma exacerbation (asthma-E). Our data provide an overview of the genetic portrait of childhood asthma and novel insights about the differences in genes important in promoting the development of asthma versus promoting the ongoing phenotype of asthma. Strong gene expression signatures that reflect clinical asthma attack status in readily sampled patient tissues provide a new opportunity for molecular subclassification and, potentially, the clinical treatment of patients with asthma.

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